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Immunomodulatory activity of heparan sulfate mimetics from *Escherichia coli* K5 capsular polysaccharide *in vitro*

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ABSTRACT

Escherichia coli K5 capsular polysaccharide (K5PS) has been used as starting material to synthesize heparan sulfate (HS) mimetics with various biological properties. This study determined the immunomodulatory activities of K5PS through the sulfation process. The immunomodulatory effects of sulfated K5 polysaccharide derivatives were evaluated *in vitro* on murine macrophages and lymphocytes. Results indicated that HS mimetics with high sulfation in the *O*-position stimulated cell proliferation of macrophages and lymphocytes, significantly enhanced cytokine secretion and upregulated the cytotoxicity of NK cells. This study suggests that high sulfation in *O*-position of HS is required for the immunomodulatory activities.

1. Introduction

Heparan sulfate (HS) is a member of the glycosaminoglycan family of polysaccharides that are a ubiquitous class of sulfated polysaccharides. HS is present in almost all tissues on the cell surfaces and extracellular matrix in the form of HS proteoglycans (HSPGs), in which its polysaccharide chains are covalently attached to the core proteins. It is involved in diverse biological processes, such as development, tissue homeostasis, angiogenesis, inflammation, and immunity (Simon Davis & Parish, 2013). The major function of the HS chains of HSPGs, a major one is their ability to interact with a wide variety of bioactive molecules, such as growth factors, proteases cytokines, and chemokines (Shute, 2012). HS polysaccharides are considered inhibitors or promoters in immune response (Chen, Wu, & Wen, 2008). The interaction between HS and immune cells enhances the processes of recognizing and arresting antigen, moving into and out of immune organs, and promoting the proliferation of lymphocytes. However, some HS polysaccharides function as immunosuppressors by blocking proinflammatory cytokines, suppressing activation of complements, and inhibiting migration and adhesion of immune cells through their interactions with chemokines (Chen, Wu, & Wen, 2008; Simon Davis & Parish, 2013). These interactions and the corresponding pathophysiological functions can be modulated by exogenous HS mimetics (Li, Zheng, Li, & Ma, 2012; Sheng, Oh, Chang, & Hsieh-Wilson, 2013).

Escherichia coli K5 capsular polysaccharide (K5PS) has been used as the starting material to synthesize HS analogs with various biological properties because of its similar carbohydrate backbone as the HS biosynthetic precursor. Chemically and/or enzymatically sulfated K5PSs possess antiproliferative, antiangiogenic, antimetastatic, and antiviral activities (Li et al., 2013). However, neither the immunomodulatory activities of the sulfated K5PSs nor their functional mechanism is clearly elucidated. The possibility that sulfated K5PSs will disturb the function of HS in immune response has not yet been investigated, although many sulfated polysaccharides have various effects on innate immune and complement system.

To clarify the immunoregulatory properties of sulfated K5PSs, this study examined the effect of different concentrations and sulfation patterns of sulfated K5PSs on macrophages and spleen lymphocytes *in vitro*. It aimed to elucidate the structure–activity relationships among the sulfated K5PSs, and further develop a new immunopotentiator.

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2. Materials and methods

2.1. Materials and reagents

Pyridine–sulfotrioxyde complex, tetrabutylammonium hydroxide, trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS, *E. coli* 0111:B4), and concanavalin A (ConA) were purchased from Sigma, USA. RPMI 1640 medium, Dulbecco's modified Eagel's (DMEM) medium, bovine serum albumin (BSA), and fetal bovine serum (FBS) were obtained from Gibco, USA. All other chemicals and solvents used were of analytical reagent grade and obtained from Sinopharm Chemical Reagent Co., Ltd., China. The enzyme-linked immunosorbent assay (ELISA) kits for interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), immunoglobulin G 1a (IgG 1a), IgG 2b, interferon-gamma (IFN-γ), and interleukin-2 (IL-2) were obtained from Shanghai Fu Sheng Industrial Co., Ltd., China.

2.2. Animals

Imprinting control region (ICR) mice (male, 20.0 ± 2.0 g) were purchased from the Laboratory Animal Center of Zhejiang Province, China (the license number: SCXK (Zhe) 2008-0033). During the experiment the mice were kept under standard laboratory conditions. All the experimental procedures were approved by the Animal Care Committee of Jiangnan University of China.

2.3. Cell lines

YAC-1 (Mouse lymphoma cell line) and RAW 264.7 cells (RAW cells, mouse macrophage cell line) were obtained from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. Cells were cultured in RPMI 1640 or DMEM supplemented with 10% FBS containing 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37 °C under humidified air with 5% CO_2.

2.4. Preparation of K5 derivatives

The capsular K5 polysaccharide was produced by *E. coli* strain 010:K5:H4 fermentation and purified from the culture supernatant, as described previously (Wang et al., 2010b; Zhang et al., 2012).

Four classes of sulfated derivatives with different degrees of *N*- or *O*-sulfation (Fig. 1) were synthesized as previously described (Leali et al., 2001). Briefly, K5-OS_1 and K5-OS_2 were obtained by direct *O*-sulfation of a single batch of K5 polysaccharide added with different concentrations of pyridine–sulfotrioxyde complex. The sample with a relatively higher degree of *O*-sulfation was named as K5-OS_2. Another one with a relatively lower degree of *O*-sulfation was named as K5-OS_1. *N*-deacetylated/*N*-sulfated K5 polysaccharide (K5-NS) was synthesized with the *N*-deacetylation step in 2 M NaOH, and then with a further step of *N*-sulfation. K5-NS was run through a cation exchange column (IR-120 H^+; Bio-Rad) at 10 °C and was neutralized with 15% tetrabutylammonium hydroxide in water. After freeze drying, the sample was dissolved in *N*,*N*-dimethylformamide, and pyridine–sulfotrioxyde complex was added to obtain *N*,*O*-sulfated K5 polysaccharide (K5-NS,OS).

2.5. Characterization of K5 derivatives

NMR spectra were recorded in D_2O (99.9%, Sigma-Aldrich) with a Bruker Advance 400 MHz spectrometer. D_2O at 4.75 ppm was used as a reference line. Molecular weight determination was performed by gel permeation chromatography with a multi-angle light scattering detector (GPC–MALS) analysis. Percentages of carbon and sulfur were determined by elemental analysis. The degree of sulfation (DS) was defined as the average number of sulfate ester groups inserted in each disaccharide unit, which was calculated in a similar way to that used by Melo et al. Disaccharide analysis was carried out using USP-based methods. Briefly, each substrate was treated with a mixture of heparinase I solution, heparinase II solution, and heparinase III solution, and incubated for 48 h at 30 °C. Strong anion exchange (SAX)-HPLC was performed to analyze the degradation products of K5 polysaccharide and sulfated K5 polysaccharide derivatives.

2.6. Macrophage activation by sulfated K5 polysaccharide derivatives in vitro

2.6.1. RAW264.7 proliferation assay

The cytotoxic effects of sulfated K5 derivatives on RAW264.7 cells were assessed using MTT assay. In brief, cells were seeded at 1×10^5 cells/mL in a 96-well plate for 24 h and then were cultured with different concentrations of sulfated K5 derivatives for 24 h or 48 h. After incubation, MTT was added to each well to a final concentration of 0.5 mg/mL. The plates were further incubated for 4 h, and then the produced formazan crystals were dissolved in DMSO. The absorbance at 570 nm was measured by microplate ELISA reader.

2.6.2. Determination of cytokine production

RAW264.7 cells were cultured for 24 h with the samples in different concentrations as above. Then supernatants were harvested

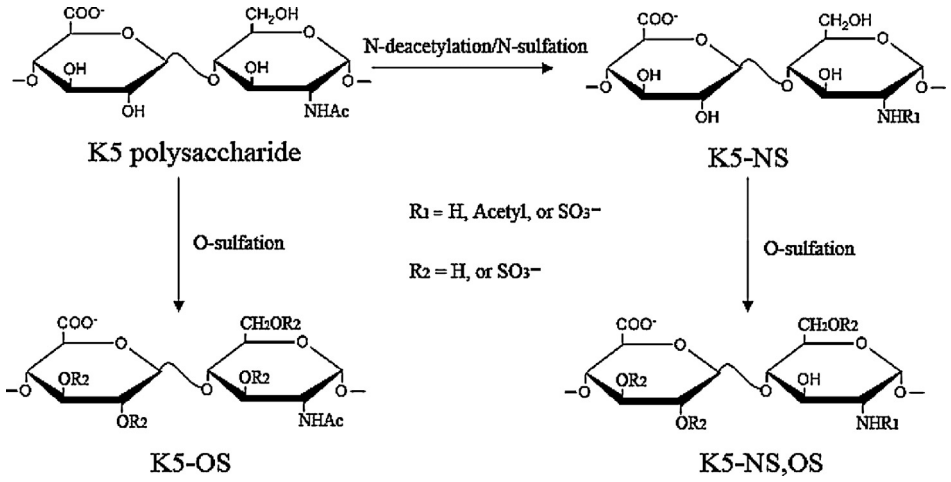

Fig. 1. Schematic illustration of the synthesis of sulfated derivatives from the K5 polysaccharide.